© by PSP Volume 27 – No. 3/2018 pages 1764-1771 Fresenius Environmental Bulletin

THE EFFECT OF ELECTROMAGNETIC RADIATION ON THE DEVELOPMENT OF SKIN ULTRASTRUCTURAL AND IMMUNOHISTOCHEMICAL EVALUATION WITH P63

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ABSTRACT

Radiofrequency (RF) waves emitted by mobile phones constitute harmful effects in cellular and molecular level. In this study, it is aimed to evaluate the effects of radio frequency of cell phone on the fetus skins of the rats during pregnancy. In this study 24 female Wistar albino rats were used. The rats were randomly divided into three groups: control, Faraday –; F (-) and Faraday +; F(+). The rats in the control group were not exposed to the mobile phone RF. The rats in F- group were exposed to the RF in special air conditioned boxes, whereas F+ group was exposed to RF in an aluminium isolated Faraday Cage. The effect of electromagnetic radiation on the prenatal development of skin was evaluated by immunohistochemical staining with p63 and as ultrastructral by Transmission Electron Microscopy (TEM). In F(+) and F(-) and control groups, differences in terms of p63 immunoreactivity was observed. Exclusively, in F(+) group, similar results were obtained to the control group in terms of p63 immunoreactivity. However, significant epidermis staining was observed in F (-) group with p63. According to the results of multiple comparison groups observed that significant a difference between two groups (p: 0,0001). In our study it has been interpreted that strong staining status for p63 immunoreactivity in the F(-) group, on the development of rat skin of mobile phone can cause significantly effects that is an indication and it was thought that advanced researches must be done on this subject.

KEYWORDS:
Electromagnetic field, Fetal skin, P63, Teratogenic effect, Radiofrequency.

INTRODUCTION

The radiofrequency (RF) generated by mobile phones causes many harmful effects on the cellular and molecular levels [1]. Both cell phones and base stations set up for communication form high-frequency electromagnetic waves that are shown to be harmful to certain tissues [2]. Within the electromagnetic wave spectrum, mobile phones in the group of radio waves are devices that send and receive low-power radio frequency (RF) signals. Currently used mobile phones operate in the frequency range of 800-1900 MHz. Mobile phones that operate at low power according to emergency communication devices operate with high power compared to cordless phones. The amount of radiation from a mobile phone depends on the strength of the signal transmitted by the mobile phone, and the effect is reduced as the distance to the mobile phone increases. [3, 4, 5]. In researches, it is stated that RF waves have negative effects on chromosomes and DNA [6, 7, 8]. The effects of RF waves on DNA suggest that mobile phones may have carcinogenic and teratogenic effects [9]. Recently, Zothansiama et al. in their study; when comparing DNA damage and antioxidant levels in blood lymphocytes among the groups, they found statistically significant results confirming the negative effects of RF [10].

In everyday, people are exposed to radiofrequency (RF) electromagnetic fields (EMFs). During the pregnancy, exposure to RF is been wondered about the side effects that may occur in the newborn. Erkut et al. have explained that bone and muscle tissue development was negatively affected due to prenatal exposure to 1800 MHz radiofrequency electromagnetic field [11]. When was investigated the effects on rat pup heart tissue of prenatal exposure to a 900 megahertz (MHz) EMF, histological examination revealed irregularities in heart muscle fibers and changes. In addition to, in electron microscopy was viewed crista loss and swelling in the mitochondria, degeneration in myofibrils and structural impairments in Z bands [12]. Furthermore, in different studies results show that a 900-MHz EMF applied in the prenatal period caused oxidative stress and pathological alterations in the liver [13], in kidney tissue [14]. In our study, the effects of 900 MHz EMF exposure in the prenatal process on the skin of rat pups were also investigated. To the best of our knowledge, a study in which the effect of EMF on skin is morphologically assessed at the level of fine structure is rare. In this study, p63 was used to investigate skin of rat pups exposed to EMF in the prenatal period. Because

several reports have explained the p63 role in epidermal development and in skin homeostasis [15]. The p53 homologue p63 produces six different isoforms that recent studies have shown that are important in keratinocyte proliferation and differentiation in the epidermis and squamous cell carcinoma [16, 17]. Absence of p63, epidermal differentiation leads to defect [18, 19]. Furthermore, p63 has also been identified as a mediator in several other processes, such as carcinogenesis [20].

Immunohistochemical analysis has showed the presence of p63; in the skin, in the basal cell layer of the squamous epithelium, and squamous mucosal surfaces, in the transitional epithelium of the bladder and in the myoepithelial cells of the breast and prostate. [21]. Reis-Filho JS et al. reported that in fibroadenoma specimens, identified p63 as a novel immunohistochemical marker of the myoepithelial / basal cell nucleusand that expressed by basal-somatic stem cells of the stratified epithelium [22, 23]. In this study, we have investigated that exposure to radiofrequency in the gestational period may cause changes in the epidermis of newborn rats and that this condition may later affect cellular differentiation and neoplasia.

MATERIALS AND METHODS

The randomised controlled study was conducted at Gaziantep University, School of Medicine, Experimental Animals Research Laboratory Gaziantep, Turkey. After approval from the Animal Experiments Ethics Committee of university, twenty four female Wistar albino rats were included. In this study, 14-16 weeks rats were used which were identified with the vaginal smears they were pregnant. The average weight of the rats was 200-250 g and the animals were kept in groups access to food and tap water ad libitum. The rats were divided into three groups as control, Faraday –; F (-) and Faraday +; F(+). Control group; unstressed and not to be exposed to EMF during the experimental period for 21 days. This group was not domiciled in the special boxes and Faraday cages. Special ventilated boxes were made for each animal of the 2nd group. This group (F-); EMF (900 MHz) received in the special ventilated boxes for 21 days (from 10:00–11:00 h) during the gestation period. Faraday + group was placed to Faraday cage which covered with aluminium. This group rats were exposed to the mobile phone RF in speech mode for 60 minutes in the Faraday cage during the gestation period (from 10:00–11:00 h).

After these applications, newborn rats with eight female rats in each group were decapitated and samples were taken from their back skin. Some of the skin specimens were placed in a 10% buffered formalin solution for immunohistochemistry and some were placed in a 2.5% glutaraldehyde solution for electron microscopy.

Light microscopic examination. Skin tissues fixed in 10% buffered formalin, and then processed in paraffin block using standard processing protocols. Sections (4-5 μm) were serially cut from a paraffin block, stained with p63 immunohistochemical procedures were performed.

Immunohistochemical (IHC) staining was performed on 4-5 μm-thick sections, after deparaffinization in xylene and rehydration through graded alcohols. Sections were kept in a microwave oven (600 watt) for 25 minutes at 95° C for the removal of the antigen mask. 3% hydrogen peroxide in 0.01M PBS, 3x5 minutes in room temperature for 10 minutes, followed by 10% normal serum blocking solution for endogenous peroxide blockade applied. 1-2 drops of the primer antibody p63 (Abcam, cat. no: ab63881 at 1 μg/ml) prepared in the appropriate dilution on the sections were dropped. Incubation was allowed in humidified boxes closed for 1 hour at room temperature + 12-24 hours at 4° C. At the end of incubation period, it was washed with buffer for 3x5-10 minutes. On the sections, 1-2 drops of the secondary antibody prepared in the diluent were dropped. In an enclosed humid box, allow to incubate for 30-60 minutes at room temperature. At the end of the incubation, wash with buffer for 3X5-10 minutes. The reaction was terminated by washing with PBS. Nuclear staining with Mayer hematoxylin or Harris hematoxylin was performed.

The sections were examined on an Olympus BX50 microscope. Immunohistochemically stained preparations prepared from each of the 8 rats in each group were examined by light microscopic evaluation and recorded with X40 magnification and 10 different areas in each preparation, with immunoreactive cells scored. For each marker, immunreactivity determined using a semiquantitative method in which the extent and intensity of staining was assesed. Percent of immunoreactive cells were obtained by counting 400-500 cells. If there is no immunoreactive cell, the score is 0, 10% positive cell; score 1, 10-50% score 2, 51-80% score 3, 80% and score over 4. The immunoreactivity intensity was semi-quantitatively assessed by the same histologist in 0.5 (Very weak), 1 (Weak), 2 (Moderate), 3 (Strong) groups. Immunohistochemical Score (IHS); [24]. as a result of multiplying immunoreactivity cell number score and immunoreactivity intensity values according to the literature. Similarly with the criteria reported by Berghmans et al. the immunoreactivities were semiquantitatively assessed by the percentage of stained cells [25].

Electron microscopic examination. Skin samples taken for electron microscopic examination were detected with 2.5% glutaraldehyde in buffer

for primary fixation and then were washed in buffer. Secondary fixation (1% osmium tetroxide in buffer) dehydration (30-100% ethanol), infiltration with transitional solvents (propylene oxide), infiltration with resin and then embedded in araldite through electron microscopic tissue tracking procedure. Resin blocks were sectioned with ultramicrotome (Leica Ultracut) at an average thickness of 60-70 nm and placed on copper grids. After contrasting the sections with lead-uracil, photographs were taken with a Jeol 1011 transmission electron microscope.

Statistical analysis. All statistical analyses were performed using the Statistical Package for the Social Sciences, version 11.5 for Windows (SPSS Inc., Chicago, IL). Kolmogorov-Smirnov test was used for normal distribution suitability, and One Way Anova test was used for group comparisons. For multiple tests Tukey tests were used, as well as descriptive statistics. In light microscopic examination, the skin epidermis thicknesses were measured from 10 different areas in each of the sections in all groups, and the averages were taken. Statistical analysis of the epidermis thicknesses of the groups was done by Brown-Forsythe method.

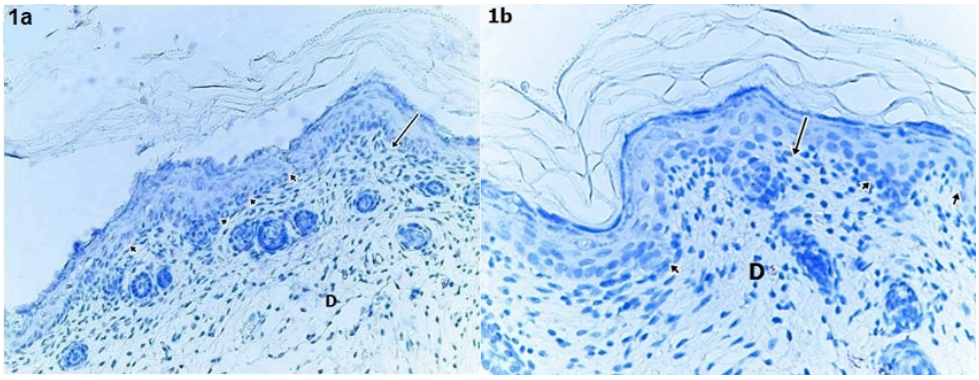

FIGURE 1
Control group is rat skin tissue. Evaluation of immunoreactivity with P63. epidermis and dermis. Arrow; epidermis, D; dermis, Arrowhead; St.Basale cells.X20, X40.

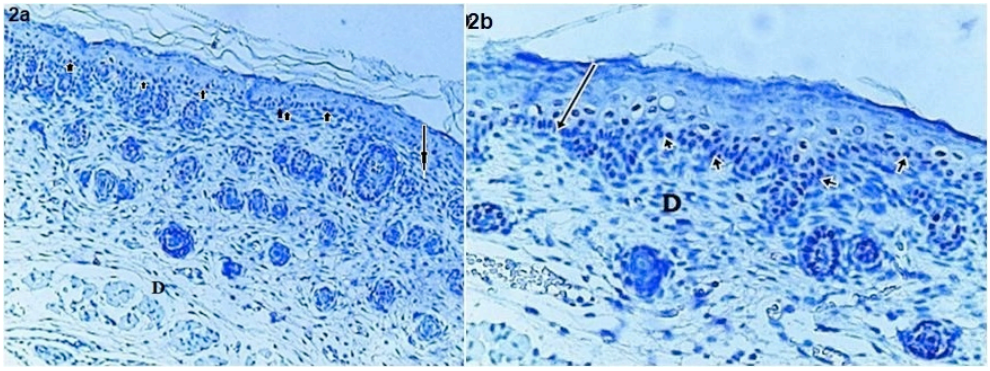

FIGURE 2
Evaluation of immunoreactivity with P63 of skin tissue of F negative rat group. Arrow; epidermis, D; dermis, Arrowhead; Commonly seen immunoreactive cells in the Stratum Basale. X20, X40.

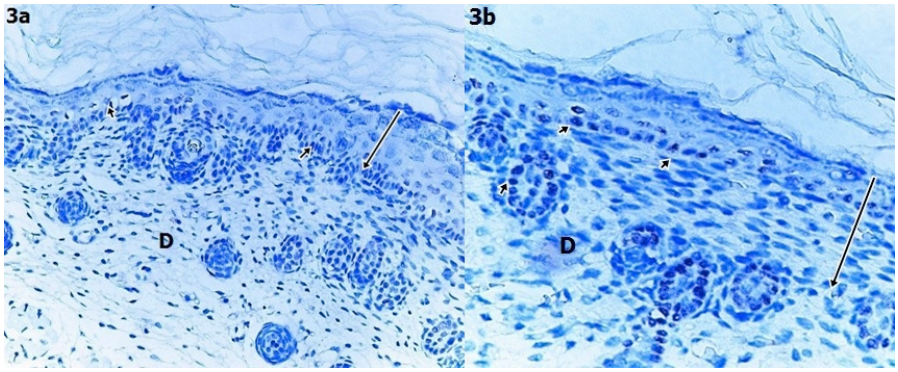

FIGURE 3
Immunoreactivity assessment of skin tissue of positive rat group with P63. Arrow; epidermis, D; dermis, Arrowhead; Immune reactive cells occasionally observed in the St. Basale. X20, X40.

RESULTS AND DISCUSSIONS

Light microscopic evaluation. In light microscopic examination, the skin tissues of the neonatal rats in the control group were evaluated in normal histological structure. When the skin epidermis layer is examined from the base to the surface, stratum basalis layer was observed in the form of a single row cell array On top of the stratum basale layer, a 6-7 cell layered stratum spinosum layer was observed, characterizing with the polygonal, densely packed cytoplasmic extensions. 1-3 layers of stratum granulosum were observed in this layer, including nuclei and keratohyalin granules in their cytoplasm (Figure 1a, b). At the surface, the stratum corneum layer contained keratin lamellae, which were seen in similar structures in all groups. F (-) (233,65 μm), F (+) (225,08 μm) and control (223,92 μm) were measured as thinner to thicker skin epidermal thicknesses of the groups (Figure 5). In the dermis layer, hair follicles and sebaceous glands were found in places. Dermis layers of all groups were similar and evaluated in normal histological structure. (Figure 2 a, b and 3 a, b). In the evaluation of immunoreactivity with P63, in Figure 2 a, b; The number of immunoreactive cells in the cells located in the stratum basalis of skin epithelium of the F (-) group is striking. Most immunostained cells were observed numerically in this group, and also immunoreactive cells were observed in the F (+) group. Staining in the control group was not apparent. As a result of the calculation of immunoreactive cells, the number of immunoreactive cells of F (-) group in numerical terms was found statistically significant.

Electron microscopic evaluation. Ultrastructural examination compared with controls in the F (-) and F (+) experimental groups exposed to radiofrequency; increase in epithelial cell nuclei and decrease in dermis connective tissue elements and structural changes were observed. It has been observed that there is a decrease in colloidal fibers and impoverishment between cells. (Figure 4).

Statistical evaluation. F (-) group had the highest epidermal thickness in terms of average of skin epidermal thicknesses. However, there was no statistically significant difference between the groups (Figure 5). One Way Anova test was used for group comparisons. As a result of the calculation of immunoreactive cells, the number of immunoreactive cells of F (-) group in numerical terms was found statistically significant. There was statistically significant difference between the groups according to the multiple comparison results (p: 0,0001). These significant differences were found between control-F negative (p: 0,0001) and F negative-F positive groups (p: 0,0001). (Figure 6).

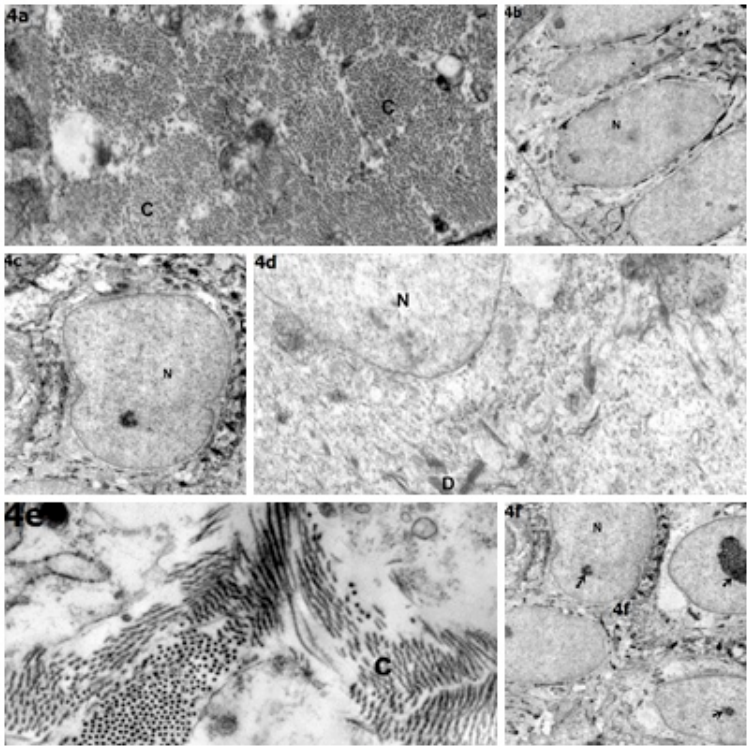

FIGURE 4

4a;b; In the skin epithel of the control group , abundant amounts of collagen fibers and echromatic nucleated glands were observed. 4c,d; In the skin epithel of the F (+) group, the cellular structure was close to the control. 4e,f; In the F (-) group of skin epithelium, increased cell nucleolus, decreased collagen fibers were observed. C; collagen fibers, N; nucleus, D; desmosome, arrowhead: Nucleolus.

TABLE 1
Descriptive statistics and Mean thickness of the rat skin epidermis layers.

	N	Minimum	Maximum	Mean± Standart Deviation	Skin epidermis Thickness
F positive	8	3,00	32,00	17,8333±10,06810	225,08 µm
F negative	8	56,00	126,00	87,0000±28,80972	233,65 µm
Control	8	0,00	1,50	0,6667±,60553	223,92 µm

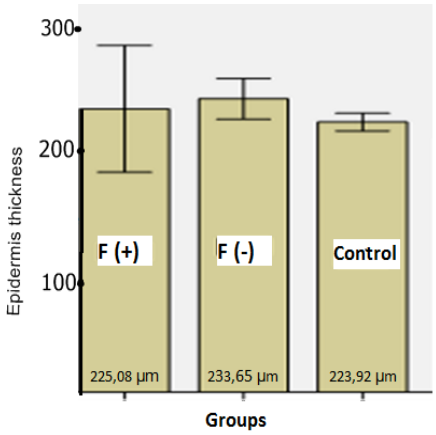

FIGURE 5
Destriptive statistics and mean of skin epidermis thickness

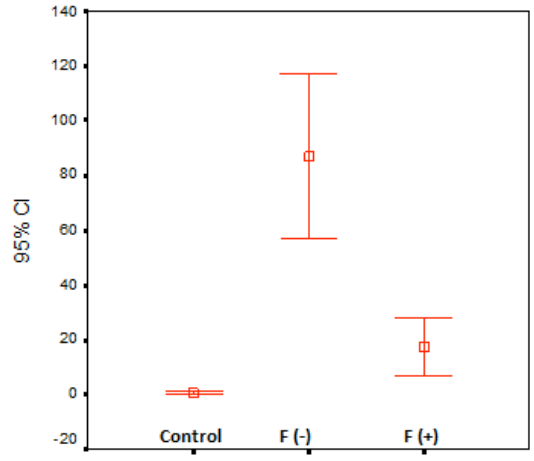

FIGURE 6
Comparison of the average number of cells showing immunoreactivity with P6

The most striking effect of EMF on living things is that it penetrates directly into the tissue. World standards for exposure to EMF are also related to thermal efficacy. It is known that weak EMF can cause dramatic thermal effects in body cells, tissues and organs. The non-thermal effects of the EMF effect continue to be discussed, but in 2011- the International Agency for Research on Cancer (IARC) – Agenda of World Health Organization (WHO), electromagnetic fields were categorized and labeled as 2B potential carcinogens. Electromagnetic fields can be dangerous not only because of cancer risk, but also because of other health problems, including electromagnetic hypersensitivity (EHS). Generated by EHS is characterized as a syndrome of non-specific multiple organ symptoms including both acute and chronic inflammatory processes, mainly in the skin and nervous systems [26,27].

In our study, it was concluded that exposure of pregnant rats to EMF may cause harmful effects of rat pups in skin tissue. On the contrary, there are studies indicating that side effects due to EMF exposure are not observed. To evaluate the possible adverse effects of multifrequency RF EMFs, Shirai et al. applied an study in which pregnant rats and their delivered offspring were simultaneously exposed to different communications signal EMFs. They stated that simultaneous whole-body exposure to EMFs did not display any adverse effects in pregnant dams, the physical, cognitive development, the fertility or the development of offspring [28,29]. In our study, the harmful effects of mobile phone radio frequencies that we frequently use in our daily lives are examined. The subject has focused on how does the exposure during pregnancy, especially in the prenatal period, affect the skin? The answer to this question has been tried to be given morphologically. It is emphasized that P63 expression is effective on epidermal differentiation and neoplasia. During embryogenesis, p63 may be the molecular switch required for initiation of epithelial stratification. p63 is primarily expressed in the basal compartment of the epidermis, that p63 has a dual role and is essential for development as well as maintenance of the epidermis [30]. In this study, it was observed that epithelial thicknesses were increased in the F (-) group directly exposed to EMF, although it was not statistically significant (Table 1). This may have increased the mitotic activity in the basal layer. In normal epidermis and epidermal appendages, almost all of the basal cells and suprabasal cells are labeled with p63. Outer root sheath cells, hair matrix cells and seboblasts are positive for p63. And p63 expression may be a marker of basal/progenitor cells -in tumors of epidermis and epidermal appendages [31]. During the early stages of skin morphogenesis, epidermal keratinocytes undergo massive proliferation and the transcription factor p63 is crucial in the development of the skin [32]. In cell proliferation, p63 functions in the regenerative capacity of basal keratinocytes. p63 appears to exert control over epidermal differentiation, which may involve control of some key signalling molecules. While studies recently have enlarged our knowledge of p63

function, much remains to be researched regarding how p63 regulates epidermal homeostasis. In addition to future efforts to identify and confirm direct p63 target genes. Individual p63 isoforms will be important to further define how p63 functions in the control of keratinocyte proliferation and differentiation [33].

In our study, in groups exposed to EMF, especially in the F (-) group, st. the basal keratinocytes showed marked immunoreactivity with p63 (Figure 2a, b). This is due to the fact that the EMF radiofrequency there may be some evidence that it influences morphogenesis by affecting the stratum basale which is the most common layer of mitosis. To distinguish human epidermal keratinocytes, epigenome profiling was performed and a catalog of genes related to epithelial development and diseases and p63-related regulatory elements was generated [34].

-p63 is consistently expressed in epidermal basal/suprabasal and adnexal basal cells. Thus, overexpression of p63 in all histological subtypes may confirm that basaloid progenitor cells are linked tumor-cell lineage and have a role in the tumorigenesis of BCC [35]. To the best of our knowledge, although there are many studies on EMF effects in the literature, a study of deep ultrastructural morphologic evaluation is not included in the literature. In our study, while in control group was observed in abundant amounts of collagen fibers, in the F (-) group exposed to EMF, irregular distribution of colloidal fibers and a decrease in some places were observed. It was also observed that there are more nucleolus in the F (-) group (Figure 4 a, b and 4 e, f). This suggests that EMF influences the morphogenesis of skin tissue, creates changes in collagen structure and may also be effective in the genetic mechanism. Indeed, in recent years, studies related to the use of the electromagnetic field for treatment have also increased. -Kang et. al. explain that an electromagnetic field is an efficient stimulation tool because it promotes bone defect healing, spite of in an unknown way [36]. Clinical effects of a multipolar radiofrequency and pulsed the electromagnetic field treatment for face and neck rejuvenation were evaluated [37]. Moraveji et. al. have shown beneficial role of EMF in inducing neural differentiation by real-time PCR analysis [38].

Radiofrequency applied with an alternating current, an electric field is generated, which achieves skin tissues, generating thermal energy. The heat is not diminished by tissue diffraction or absorption by epidermal melanin and is then suitable for treatment of all skin types [39]. It has been said that nuclear envelope-associated genes as signifiicant targets mediating p63-regulated gene expression programme in the epidermis [40]. Recently studies mention that an increase in laptop user's skin temperature will have a destructive effect on skin permeability [41]. Sasaki et al. emphasize that increasing the potential risk of skin burn of the temperature rise in millimeter-wave and terahertz-wave exposure [42]. Perhaps, it can be assumed that the EMF radiofrequency may be effecting differently depending on the dose and duration.

CONCLUSIONS

The effect of the electromagnetic field on the tissues is still being discussed. However, it is widely accepted that the intensity of the radiofrequency and the duration of exposure are important in the formation of harmful effects of EMF. In our study, in light microscopic level, increase of immunoreactive cells in F (-) group and changes in cell and intercellular structure at electron microscopic level; was interpreted as an indication that the mobile phone may cause harmful effects on different levels of rat skin development and it was concluded that further research should be done in this regard. As a result of a general overview; the fact that EMF is effective in the morphogenesis and differentiation process of the skin tissue in the prenatal period may have a strong side effect in the postnatal period.

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Received: **28.09.2017**
Accepted: **30.12.2017**

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